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An Unusual Tocopherol Isomer with an O-Methyl Group from *Euglena gracilis*[1]

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A compound, isomeric with α-tocopherol, has been isolated from *Euglena gracilis* strain Z, and a method has been devised for the small-scale purification and quantitative assay of this isomer using gas–liquid chromatography. The new compound was present in another strain of *E. gracilis*, but could not be isolated from any of the other photosynthetic microorganisms, plants, or plant oils which were examined. Chemical and spectral studies of the acetyl derivative of the new compound showed the presence of the following substituents around a benzene ring; one methoxy, one acetoxy, two methyls, one proton, and a phytyl group. Since quinonoid compounds are frequently those with *para* orientation of the oxygens, the six possible structural isomers of the acetyl derivative in which the methoxy and acetoxy groups were oriented *para*, were synthesized. Gas chromatography and spectral information demonstrated that the acetyl derivative of the tocopherol isomer had a structure which was very similar, although not identical, to any of the synthetic compounds.

INTRODUCTION

The tocopherols, which are biosynthesized by most photosynthetic tissues but not by mammals, belong to a family of structurally similar lipids related to the terpenoid quinones. Eight compounds, which differ in the degree and position of methyl substitution of the aromatic nucleus (tocopherols) and in the degree of unsaturation of the isoprenoid side chain (tocotrienols), have been isolated from plant sources (*1*).

There are two types of quinones which are structually related to the tocopherols—the tocopherol quinones and the plastoquinones. These compounds have a widespread natural distribution and have been implicated in photosynthesis and electron transport. The tocopherol quinones, which may be derived from the corresponding tocopherols by chemical oxidation with gold chloride (*2*), also occur in plants (*3*, *4*) and blue–green algae (*5*). The plastoquinones contain the 2,3-dimethyl-1,4-benzoquinone nucleus substituted with a polyprenyl side-chain. Plastoquinone-A contains a side-chain of nine unsaturated isoprene units in contrast to the tocopherol quinones which contain four saturated isoprene units, one substituted with a hydroxyl group. There are also related plastoquinones (plastoquinones-B, -C, and -Z) which contain a nonaprenyl side-chain modified in various ways (*6*). A plastoquinone which contains four unsaturated isoprene units, has been isolated from horsechestnut leaves, but has not been reported from other natural sources (*7*). In addition, a plastoquinone containing a side-chain consisting of three unsaturated isoprene units has been isolated from spinach chloroplasts (*8*).

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In preliminary experiments directed to a study of tocopherol biosynthesis, lipid fractions of *Euglena gracilis* strain Z were examined. In addition to α-T[3] itself, we isolated and purified a material with the same molecular formula. Since this material had several similarities to α-T, it was called "α-tocopherol isomer" (I). This paper describes the purification, assay, general properties, and possible structure of this lipid. It now appears not to be a true tocol derivative but rather an example of a new isoprenoid aromatic derivative.

MATERIALS AND METHODS

Materials

The microorganisms were obtained from the American Type Culture Collection (ATCC). The strain of *Euglena gracilis* used routinely was *E. gracilis* strain Z (ATCC 12716). The other organisms used were: *E. gracilis* Klebs var. bacillaris (ATCC 10616), *Chlorella vulgaris* (ATCC 11468), *Chlamydomonas pseudagloe* (ATCC 12235) and *Ochromonas danica* (ATCC 30004). The medium used for the growth of *E. gracilis* strain Z, *E. gracilis* var. bacillaris, *C. pseudagloe* and *Chlorella vulgaris* consisted of: 1.0 g beef extract (Difco), 2.0 g Bacto-Peptone (Difco) 2.0 g yeast extract (Difco), and 1.0 g sodium acetate in 1 liter of water (*9*). The medium used for the growth of *O. danica* was as follows: 0.3 g yeast extract (Difco), 0.7 g Bacto-Tryptone (Difco), and 3.3 g glucose per liter of water, with the final pH adjusted to 7.2.

Reagent grade solvents were used without further purification except that petroleum ether was redistilled and the fraction boiling between 40 and 60°C was used for all experiments. Pyridine was stored over potassium hydroxide pellets.

The alumina used for column chromatography was Brockmann Grade I Woelm acid (anionotropic) aluminium oxide (Alupharm Chemicals, New Orleans, La). The Brockmann Grade III used for the separation of the lipids from *E. gracilis* was prepared as follows. The Grade I alumina was placed in a mortar and covered with petroleum ether; water (6% of the weight of the alumina) was added dropwise and the mixture was vigorously agitated for 5 min until the water and alumina were thoroughly mixed. Silicic acid for column chromatography (200–325 mesh) was purchased from the Clarkson Chemical Company, Williamsport, Pa. The silica gel for thin-layer chromatography was Silica Gel G obtained from Brinkmann Instruments Inc., Westbury, NY; it was washed with chloroform before the plates were prepared. The thin-layer plates, which were impregnated with Rhodamine 6G, had a thickness of 250 μ and each plate was washed in the solvent system to be used before application of the compounds. Samples for preparative thin-layer chromatography were applied to the plates by means of a Radin–Pelick sample streaker (Applied Science).

The column packings for gas–liquid chromatography and combined gas chromatography–mass spectrometry were prepared as described by Wells et al. (*10*), or were obtained from Applied Science Laboratories, State College, Pa. The columns used routinely were 6 ft × ¼ in i.d. glass columns packed with 1½% OV-1 (coated on 80–100 mesh Gas-Chrom S) or 3% OV-17 (coated on 60–80 mesh Gas-Chrom Q).

Standard samples of (+)-α-tocopherol acetate, (±)-α-tocopherol acetate, and (+)-α-tocopherol were purchased from Distillation Product Industries; samples of phytol and isophytol were obtained from Nutritional Biochemical Corporation, Cleveland,

[3] Abbreviations used: α-T, α-tocopherol; α-TAc, α-tocopherol acetate; CP, cholesterol propionate; TMSi, trimethylsilyl.

Ohio. Cholesterol *n*-propionate (Aldrich Chemical Company, Inc., Milwaukee, Wis. was recrystallized from methanol.

Methods

Growth of the organisms. Cultures of *E. gracilis* strain Z, *Chlorella vulgaris* and *Chlamydomonas pseudagloe* were maintained on agar slopes of the previously described acetate medium, solidified with 2% Bacto-Agar (Difco). *E. gracilis* var. bacillaris and *O. danica* were constantly maintained as liquid cultures. All cultures were examined periodically under the microscope to observe any bacterial or fungal contamination. For extraction of the tocopherols the organisms were grown for 8 days at room temperature in the presence of fluorescent lighting in 2.8-liter Fernbach flasks containing 600 or 800 ml of medium. Each flask was shaken daily for 30 sec. In a typical experiment, six flasks of *E. gracilis* (4.8 liters of growth medium) yielded 25 g wet weight of cells.

Purification of the tocopherol isomer. Cells of *E. gracilis* were obtained by centrifugation in the Sorvall Continuous Flow Centrifuge at a speed of 12,500 rpm and were then washed with distilled water. In initial experiments, extraction of lipids was carried out by the method of Threlfall and Goodwin (*11*). However, this procedure was rather tedious for large volumes and a new procedure was developed. The washed cells were extracted with cold acetone in a Waring Blendor for 1 min. The white cell debris was filtered from the dark-green acetone extract which contained the tocopherols and chlorophyll. The crude lipid extract (about 250 mg from 25 g of cells) was partially resolved into its lipid components by chromatography on a column (35 g, 22 × 1.5 cm) of Brockmann Grade III alumina. The column was eluted with 10 column volumes of each solvent (350 ml). The first fraction, eluted with petroleum ether, contained the carotenes; 1% and 3% diethyl ether in petroleum ether eluted the α-tocopherol isomer (I) and α-tocopherol, respectively. The column was eluted at 4°C since room temperature caused the low-boiling solvents to evaporate and produce air bubbles in the column.

After gas chromatographic assay of the column fractions for the α-tocopherol isomer as its trimethylsilyl derivative as described below, the 1% ether in petroleum ether fraction from the alumina column (15 mg) which contained I, was acetylated with a 1:1 mixture of pyridine–acetic anhydride. The mixture was allowed to stand at room temperature for 30 min, then extracted with ether. The combined ether extracts were washed first with water, then with 6 *N* hydrochloric acid, and finally with water. The ethereal solution was dried (anhydrous sodium sulfate), filtered, and evaporated. The crude sample of the tocopherol acetate isomer (IAc) (10 mg) was partially purified by elution with benzene (100 ml) from a silicic acid column (10 g, 29 × 1 cm). IAc was further purified by thin-layer chromatography in chloroform; since IAc co-chromatographed with α-tocopherol acetate[3] in this solvent system, spots of α-TAc were run as markers. The compounds were visualized under uv light (254 nm), and the appropriate band was scraped off the plate and eluted from the silica gel with ethyl acetate. A second preparative thin-layer chromatography plate, run in chloroform : benzene, 1:1, completed the purification. α-T was purified as its acetate by the same procedure.

In a typical extraction, 25 g wet weight of cells gave approximately 1 mg of both α-TAc and IAc. The recovery of a known amount of α-T was tested at each purification step; the overall recovery of α-T after the final purification step (i.e. after acetone treatment, alumina column, acetylation, silicic acid column, repeated thin-layer chromatography) was 65%.

Gas chromatographic assay of the tocopherols. The trimethylsilyl derivatives of α-T

and I were prepared according to Sweeley et al. (*12*). In some cases the compounds were trimethylsilylated by the addition of bis-(trimethylsilyl) acetamide (Supelco Inc., Bellefonte, Pa).

The following gas chromatographic conditions were routinely used for the quantitative and qualitative analysis of the TMSi or acetyl derivatives of the tocopherols:

Instrument: F & M Model 402, with flame ionization detector
Column temperature: 235°C
Injector temperature; 250°C
Detector temperature: 250°C
Column: 6 ft × ¼ in glass, 1 ½% OV-1 coated on Gas-Chrom S (80–100 mesh)
Carrier gas flow rate: 30 ml/min.

For assay of either the TMSi or acetyl derivative, cholesterol propionate[3] was used as an internal standard. This compound was stable at the temperatures used and had an appropriate gas chromatographic retention time. For the TMSi method, an aliquot from the mixture containing the tocopherol was placed in a sample vial (2-ml volume) and the solvent was evaporated under a stream of nitrogen. The silylating reagent (10–50 μl) was then added to the dry sample with a known amount of cholesterol propionate. After 10 min an aliquot of the sample was injected into the gas chromatograph. A comparison of the area under the peak corresponding to the tocopherol derivative with that of the internal standard, established the amount of the tocopherol present in the sample. An experimentally determined correction factor was used to account for the difference in detector response to the tocopherol derivative and cholesterol propionate. This novel approach to the quantitative assay of a tocopherol (α-T or I) in either a pure sample or a mixture is suitable for the determination of a small amount of a tocopherol (1 μg or more) with a precision of 5%.

Spectral methods. The following conditions were used for combined gas chromatography–mass spectrometry:

Instrument: LKB 9000
Columns: 6 ft × ¼ in. glass, 3% OV-1 or 3% OV-17 coated on Gas-Chrom Q (60–80 mesh)
Ionization potential: 70 eV or 15 eV
Multiplier: 1.9
Trap current: 60 μA

High-resolution mass spectra were obtained through the courtesy of Dr C. Hignite, Massachusetts Institute of Technology, using a CEC-110B mass spectrometer.

The nuclear magnetic resonance spectra were run in carbon tetrachloride with tetramethylsilane as the reference. The 60-MHz spectra were obtained by Dr. G. Schultz at the Gulf Research Laboratories, Pittsburgh, Pa. on a Varian 60-MHz spectrometer. Dr. F. Sprecher (Mellon Institute, Pittsburgh, Pa) kindly provided the spectra at 100 MHz using a microcell and degassed carbon tetrachloride, and Dr. Ted Lindstrom, University of Pittsburgh obtained the 90-MHz spectra with a Bruker spectrometer. Infrared spectra were all taken in dry carbon tetrachloride on a Perkin–Elmer 237B spectrometer; ultraviolet spectra were obtained with a Zeiss PMQ II ultraviolet spectrometer in absolute ethanol. The instrument used for the optical rotation studies was a Cary 60 recording optical rotatory dispersion–circular dichroism spectrometer; all samples were dissolved in spectranalyzed *n*-heptane at a concentration of approximately 5 mg/ml. Radioactive samples were dissolved in hexane and counted

in 0.4% diphenyloxazole in toluene on Packard Tri-Carb scintillation spectrometers, models 3310 and 3375.

Catalytic hydrogenation. For catalytic hydrogenation of IAc and the synthetic dimethylphytyl-1-methoxy-4-acetoxybenzenes, the sample (2–10 mg) was dissolved in 10 ml cyclohexane : ethanol (1:1) and platinum oxide catalyst (Engelhard Industries, Newark, NJ) (approximately 5 mg) was added. Hydrogen gas was bubbled into the reaction flask and the sample was hydrogenated with stirring for 1 hr. After filtration of the catalyst and evaporation of the solvents, a colorless oil was obtained.

RESULTS

Evidence for the Existence of a Tocopherol Isomer

A good preliminary separation of I and α-T was achieved by chromatography of the total lipid extract on alumina. Compound I eluted with 1% ether in petroleum ether, whereas the more polar α-T required 3% ether in petroleum ether. Numerous other components, including plastoquinone-A, were also present in the 1% ether–petroleum ether fraction; the complexity of these fractions is indicated by the results of thin-layer and gas-liquid chromatography (Fig. 1). Since I appeared to be quite unstable

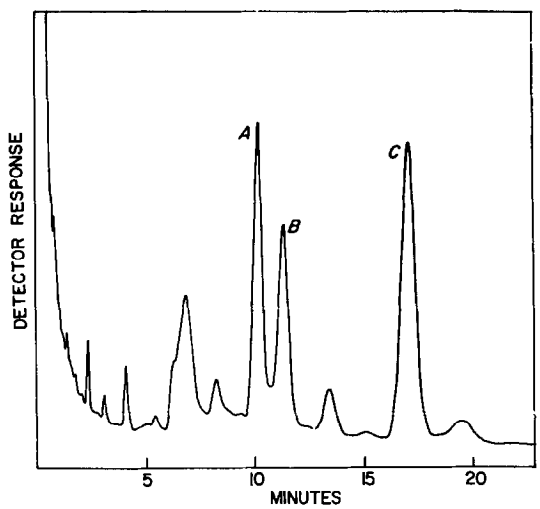


FIG. 1. Gas–liquid chromatography of the trimethylsilylated 1% ether–petroleum ether fraction from the alumina column. Standard α-T was added prior to formation of the TMSi derivative. *A*, TMSi derivative of isomer; *B*, TMSi derivative of α-T; *C*, cholesterol propionate as internal standard.

to thin-layer chromatography in chloroform or benzene and could not be recovered from the plates, the total material in the 1% ether–petroleum ether fraction was subjected to acetylation; the acetate mixture was then partially purified by passage through a column of silicic acid. Some impurities were still present in the column effluent; fortunately, IAc was stable to thin-layer chromatography and could be quantitatively recovered from thin-layer plates. After two plates the isomer acetate was pure according to the following criteria: (a) a single spot on thin-layer chromatography in three solvent systems—100% chloroform, chloroform : benzene, 1:1, and cyclohexane : benzene, 2:3; (b) a single peak on gas–liquid chromatography on two columns—3% OV-1 and 3% OV-17.

Acetylation of the material present in the 3% ether–petroleum ether fraction from the original alumina column, then silicic acid treatment and thin-layer chromatography as just described for the purification of IAc, gave pure samples of α-TAc.

α-TAc and IAc co-chromatographed on thin-layer chromatography. However, the two compounds separated on gas–liquid chromatography (Fig. 2), as did the two trimethylsilyl ethers (Fig. 1). On OV-1 and OV-17 columns, the TMSi or acetyl derivatives of I had shorter retention times than did the corresponding derivatives of α-T, again indicating that I was less polar than α-T.

The new isomer was shown to be a naturally occurring compound and not an artifact of the isolation procedure by the following experiments. A standard sample of α-T was

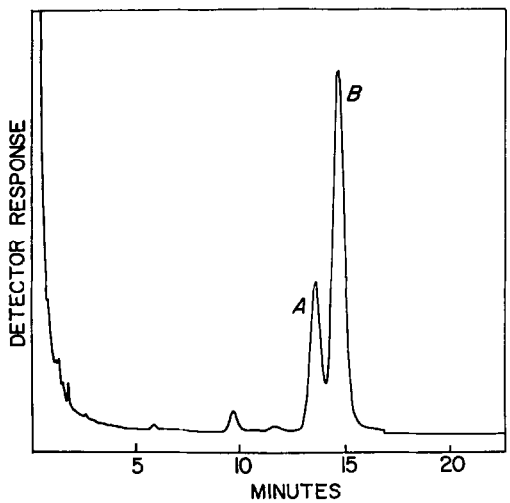


FIG. 2. Gas–liquid chromatography of α-TAc and the acetate of the new compound. The separation was carried out on a 6 ft, 1½% OV-1 column at 235°C. *A*, IAc; *B*, α-TAc.

subjected to the acetone treatment and the alumina column chromatography; none of the isomer was detected and the α-T was quantitatively recovered from the column. Therefore, I did not originate from α-T during acetone treatment or alumina chromatography. When an acetone extract without chromatographic purification from *Euglena gracilis* was gas chromatographed as the trimethylsilyl derivative, the TMSi ethers of both α-T and I were identified on the chromatogram, showing that both compounds were present in the lipid extract. The radioactive tracer experiments (see below) in which labeled precursors were equally incorporated into both α-T and I proved that I could not have arisen from an external source such as impure solvents. Final proof for the natural occurrence of I came from experiments with other photosynthetic protists, plants, and plant oils. In several cases, using the same techniques, solvents, etc. (see Distribution of Tocopherol Isomers in Nature), although α-tocopherol was present, the isomer could not be detected again showing that I is a naturally occurring component of *E. gracilis* lipids and is not an artifact of the isolation procedure.

The isomer was routinely isolated in the pure form as its acetyl derivative. Several unsuccessful attempts were made to isolate I as the free phenol, including thin-layer and column chromatography on Sephadex LH 20. However, the free phenol was unstable to thin-layer chromatography and when isolated from the Sephadex column was still

very impure. Attempts to hydrolyze IAc to the phenol under the same conditions which hydrolyzed α-TAc to α-T, (reflux for 3 hr with 5% alcoholic NaOH in an atmosphere of nitrogen) were unsuccessful. It appeared that IAc was being hydrolyzed under these conditions, but that the free phenol, once formed, was unstable to the conditions of hydrolysis. Milder reaction conditions were used (lithium aluminum hydride or sodium methoxide) but in every instance any conditions that removed the acetate group also destroyed I.

Distribution of Tocopherol Isomer in Nature

In addition to *E. gracilis* strain Z, the four photosynthetic protists listed in Table 1 were examined for their content of α-T and the isomer. *E. gracilis* var. bacillaris contained both I and α-T in amounts similar to *E. gracilis* strain Z. The other three photosynthetic protists examined—*Ochromonas danica*, *Chlorella vulgaris*, and *Chlamydomonas pseudagloe*—contained α-T but none of the isomer could be detected. The sensitivity of the method was such that as little as 0.01 μmole would have been detected by gas chromatography.

TABLE 1

THE OCCURRENCE OF α-TOCOPHEROL AND ITS ISOMER IN SOME PHOTOSYNTHETIC HIGHER PROTISTS

Organism	Wet weight of cells (g)	α-Tocopherol (μmoles)[a]	Isomer (μmoles)[a]
E. gracilis strain Z	48	5.1	3.5
E. gracilis var. bacillaris	32	7.0	3.1
Ochromonas danica	28	3.5	0[b]
Chlorella vulgaris	51	0.8[c]	0[b]
Chlamydomonas pseudagloe	33	0.7	0[b]

[a] Determined by gas chromatographic assay.
[b] Either completely absent or present in quantities of less than 0.01 μmole.
[c] Extraction of lipid may have been incomplete.

If I had a general distribution in nature, it might be expected to occur in spinach leaves, which are known to be an excellent source of α-T. Therefore, spinach leaves were blended for 1 min in the Waring Blendor with acetone: ethyl acetate: water (1 : 1 : 1). After evaporation to dryness, the green organic phase was applied to an alumina column and the amounts of I and α-T were determined by gas chromatography of the TMSi derivatives. Although none of the isomer was detected, α-T was present in large quantities (275 μg/100 mg total lipid or 550 μg/50 g spinach leaves).

Four plant oils (wheat germ oil, cottonseed oil, coconut oil, and safflower oil) were saponified and the nonsaponifiable fraction was examined for the content of I. Although α-T was isolated from all of the plant oils, the isomer could not be detected.[4]

[4] There is some doubt whether compound I would have survived the saponification step. Based on the failure to recover any identifiable material from hydrolysis of IAc, we consider that these new materials are relatively unstable. Paradoxically, in some experiments, I was recovered from crude extracts of *E. gracilis* (as shown by gas chromatography of the TMSi derivative) after saponification.

Comparative Biosynthesis of I and α-tocopherol in E. gracilis

Two radioactive tracer experiments were conducted with *E. gracilis* strain Z. In the first, 100 μCi of [U-^{14}C]L-phenylalanine was added to 3.5 liters of growth medium on the second day of growth. α-T and I were purified to constant specific activity and the results are presented in Table 2; there was an approximately equal incorporation of

TABLE 2

INCORPORATION OF [U-^{14}C]PHENYLALANINE AND [3-^{14}C]TYROSINE INTO THE TOCOPHEROLS OF *E. gracilis* STRAIN Z

	From [U-^{14}C]phenylalanine		From [3-^{14}C]tyrosine	
	Specific activity (dpm/μmole)	Incorporation (%)	Specific activity (dpm/μmole)	Incorporation (%)
Carotenes (crude)[a]	1,900		1,230	
α-Tocopherol acetate	89,800	0.09	319,400	0.10
α-Tocopherol acetate isomer (IAc)	82,100	0.09	380,420	0.10

[a] μMoles of carotene were calculated from the weight of the petroleum ether fraction from the alumina column.

radioactivity into both α-T and I, 0.09%. The β-carotene fraction was not rigorously purified but the incorporation of radioactivity into this fraction was low. Since the incorporation of ^{14}C into the β-carotene fraction was low, it may be presumed that the level of incorporation into the isoprenoid portion of the tocopherol was also low, and that the majority of radioactivity resided in the aromatic nucleus of α-T and I. It should be noted that some breakdown of the aromatic amino acids to acetoacetate would be expected (*13*).[5]

In a parallel experiment, 500 μCi of [3-^{14}C]DL-tyrosine was added to *E. gracilis* cultures. The results presented in Table 2 show that there was approximately equal incorporation of ^{14}C into both α-T and I (0.10%) [Carbon atom 3 of tyrosine is presumed to give rise to one of the aromatic methyl groups in the α-T molecule by analogy with the experiments of Whistance and Threlfall (*14*)].

Structural Information on the Tocopherol Isomer

In our preliminary investigations of the structure of the new compound we determined the mass spectra of the acetyl and TMSi derivatives (*15*, *16*). It was, in part, the general similarities of these spectra with those of the corresponding derivatives of α-T which suggested we were dealing with an isomeric situation. For the spectra of the two TMSi ethers, the most striking feature was that the same fragment ions were present in each spectrum, and that there were no major ions present in one spectrum that were absent in the other. The molecular ion (m/e 502) was the same for both I and α-T, and in each case it was also the base peak.[6] However, there were differences in the intensity of certain peaks—for example, m/e 237 had a relative intensity of 81.8% of the base peak in the α-T-TMSi spectrum, whereas its relative intensity was only 25.9% for I-TMSi.

[5] Contrary to the findings of the present study, Whistance and Threlfall (14) examined the incorporation of radioactivity from DL-[β-^{14}C]tyrosine into the isoprenoid quinones and chromanols of *E. gracilis* and found that extensive randomization of the label occurred; the β-carotene was also highly labeled in their experiment.

[6] In the mass spectra, M^+ refers to the molecular ion; m/e is the mass to charge ratio. The figures in parentheses refer to the relative intensities of the fragment ions.

The mass spectra of α-TAc and its isomer (Fig. 3) were very similar; they produced the same molecular ion (m/e 472) and the same fragment ions. However, there were important differences in the relative intensities of some fragment ions, which are shown in Table 3. For example, in the spectrum of α-TAc, the base peak was at m/e 430 and

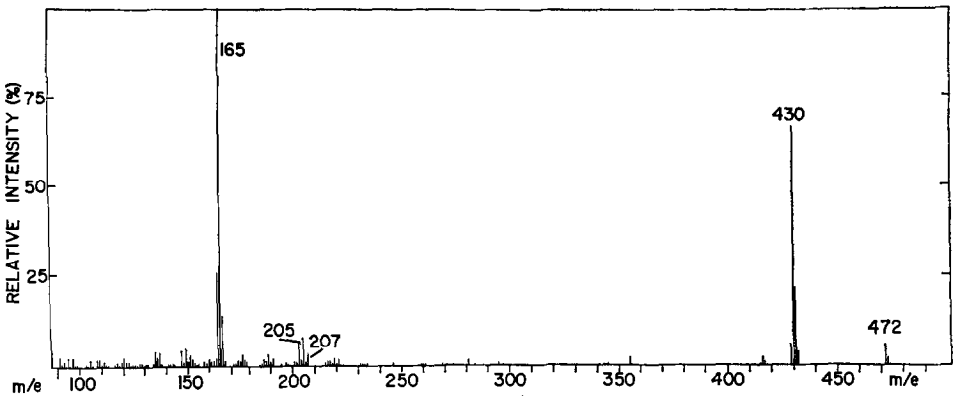


FIG. 3. Mass spectrum of IAc obtained by combined gas chromatography–mass spectrometry.

TABLE 3

RELATIVE ION INTENSITIES FOR THE MASS SPECTRUM OF α-TOCOPHEROL ACETATE AND ITS ISOMER

m/e	Relative ion intensity	
	α-Tocopherol acetate	Isomer
472	14.2	8.7
430	100.0	100.0
207	28.4	5.5
205	8.2	8.2
165	87.8	150.6
152	1.7	2.0
135	4.9	4.1
43	1.7	17.3

the second most intense ion was at m/e 165; the situation was reversed for the isomer—the base peak was m/e 165 and m/e 430 was the peak of second highest intensity. High resolution mass spectrometry yielded the following fragment ions:

m/e 472.392	(calculated for $C_{31}H_{52}O_3$,	m/e 472.39161)
430.379	$C_{29}H_{50}O_2$,	430.38105)
207.104	$C_{12}H_{15}O_3$,	207.10212)
205.123	$C_{13}H_{17}O_2$,	205.12285)
165.092	$C_{10}H_{13}O_2$,	165.09155)

The close similarity between the mass spectra of the derivatives of α-T and I indicated that I was an isomer of normal α-T, with one free phenolic hydroxyl group and a similar structure.

The ultraviolet spectrum of IAc (λ_{max} at 276 nm, shoulder at 285 nm) was slightly different from that of α-TAc (λ_{max} 285 nm, shoulder 280 nm); the spectrum of IAc was unchanged on the addition of dilute alkali or sodium borohydride. The infrared spectrum of IAc was very similar to that of α-TAc; the absorption wavelengths of the major bands were as follows: λ_{max} (CCl_4) : 3.38 μ, 3.42 μ, 3.49 μ, 5.67 μ, 6.80 μ, 7.30 μ, 8.30 μ. This spectrum confirmed the presence of the acetyl carbonyl group (5.67 μ) and the ether bond (8.30 μ). The specific optical rotation of IAc at 330 nm, $[\alpha]_{330}$, was +7.1 deg cm^2 dg^{-1}, which is of the same order of magnitude as $[\alpha]_{330}$ for α-TAc (+10.0 deg cm^2 dg^{-1}).

Since it was suspected that IAc contained an aliphatic double bond, a sample was catalytically hydrogenated. The product was a compound with a molecular weight two units higher than the starting IAc; this indicated that there was one aliphatic double bond in IAc. The mass spectrum showed the following fragment ions[5]: M^+ 474 m/e (4.7), 432 (100.0), 165 (48.0), 152 (1.8), 151 (4.4), 43 (12.3).

When IAc was incubated at room temperature for 3 hr with *m*-chloroperbenzoic acid in methylene chloride, a new compound was derived from IAc. A comparison of the mass spectrum of the product with that of IAc (Fig. 3 and Table 3), showed that the molecular weight of this new compound (488) was 16 mass units higher than that of IAc but that the fragmentation pattern was very similar to that of IAc. The mass spectrum showed the following fragment ions: M^+ 488 m/e (7.3), 430 (4.4), 207 (3.6), 205 (2.6), 203 (22.2), 165 (100.0), 43 (28.7). Hence, an epoxide of IAc had been formed at a double bond of an isoprene unit.

These spectral data show that IAc is related to α-TAc but do not give any indications of the differences in structure. The nuclear magnetic resonance spectrum of IAc (summarized in Table 4) showed striking differences in the structures of IAc and

TABLE 4

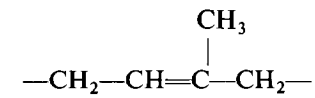
SUMMARY OF THE CHEMICAL SHIFTS FOR THE NUCLEAR MAGNETIC RESONANCE SPECTRA OF IAc AND SYNTHETIC COMPOUNDS

Compound	Chemical shift, τ			
	Aromatic H	Acetyl-CH_3 Aromatic-CH_3	$-OCH_3$	Aromatic-CH_2-
IAc	3.63	7.84(s)[a], 7.97(s) 8.07(s)	6.32(s)	6.97(d)
1	3.55(s)	7.85(s), 8.07(s)	6.42(s)	6.78(d)
2	3.54(s)	7.84(s), 8.02(s) 8.06(s),	6.42(s)	6.73(d)
3	3.43(s)	7.82(s), 7.98(s) 8.11(s)	6.39(s)	6.70(d)
5	3.56(s)	7.80(s), 7.96(s) 8.04(s)	6.27(s)	6.74(d)
6	3.43(s)	7.80(s), 7.84(s) 7.86(s)	6.38(s)	6.90(d)

[a] (s) = singlet; (d) = doublet.

α-TAc. The major features of the spectrum of IAc were: (a) the three-proton signal at $\tau = 6.32$ which represented a methoxyl group; (b) only 3 singlets (of 3 protons each) in

the region of aromatic methyls and acetyl methyl signals, showing that there were only two aromatic methyl groups in IAc; (c) an aromatic proton signal at $\tau = 3.63$. In addition, the spectrum indicated the presence of the following structural element:



A vinyl methyl group was identified as a broad singlet at $\tau = 8.38$; there was also a vinyl proton ($\tau = 4.9$) and a vinyl methylene (doublet, $\tau = 6.97$). This methylene was adjacent to the vinyl proton but not adjacent to another grouping with a free proton, therefore, it appeared that the vinyl methylene at $\tau = 6.97$ was attached to the aromatic ring. The other vinyl methylene group was not clearly defined but there was evidence for it in the region $\tau = 7.8$ to 8.1, since the integration of the two methyl groups at $\tau = 7.79$ and 8.07 gave eight protons instead of the expected six. A more detailed spectrum (100 MHz) revealed that the vinyl methyl group gave a secondary smaller signal at a higher τ value, which may indicate that IAc is a mixture of two components, the majority with a *trans* arrangement about the double bond and a smaller amount of the *cis* isomer.

The technique of double resonance confirmed the presence of the isopentene group. When the vinyl proton was decoupled, the doublet representing the vinyl methylene at $\tau = 6.97$ collapsed into a singlet.

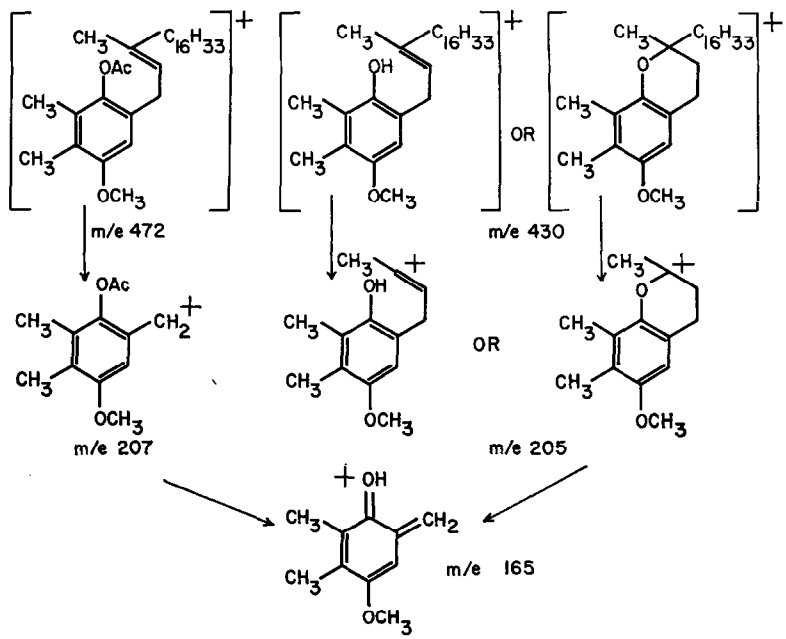
Lack of Vitamin E Activity in Compound **1**

The experiment involved three groups of young male rabbits (400–500 g); group I (four rabbits) was fed a diet containing α-TAc (2 mg/kg body weight/day); group II (three rabbits) was fed a diet which contained no α-TAc; group III (three rabbits) was fed a diet which contained no α-TAc but instead contained synthetic compound **1** (2 mg/kg body weight/day). The rabbits in group I functioned normally for the duration of the experiment (50 days), whereas all rabbits in groups II and III rapidly developed muscular dystrophy (as monitored by a sharp increase in creatine/creatinine ratio in the urine) and died within 30 days. There appeared to be no difference in the rate at which rabbits from groups II and III developed dystrophy. Thus, we conclude that 1-methoxy-2,3-dimethyl-4-acetoxy-5-phytylbenzene is not active as a vitamin E factor for the prevention of muscular dystrophy in rabbits. Since such a small amount of the natural tocopherol isomer was available (approximately 5 mg) the biological activity was not examined. The synthetic compound, **1**, was tested for vitamin E activity as a model compound which contained all the functional groups which IAc also contained.

DISCUSSION

The major structural features of the new compound from *Euglena gracilis* have been defined by the present work. The similarities in the mass spectra of the derivatives of the new compound and those of α-T established a general structural relationship; clearly, I contained a phenolic group and an isoprenoid side chain, $C_{20}H_{39}$. However, the nuclear magnetic resonance spectra and chemical studies demonstrated the absence of a chroman ring system in the new compound. The formation of an epoxide on incubation of IAc with *m*-chloroperbenzoic acid, and the addition of two hydrogen atoms on catalytic hydrogenation, confirmed the presence of one aliphatic double

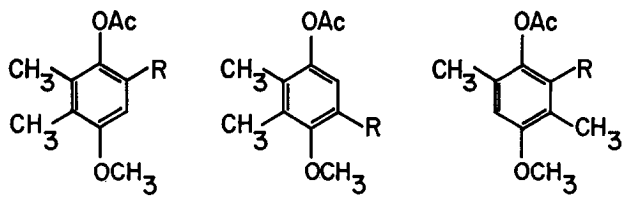
bond in the isoprenoid side-chain. The nuclear magnetic resonance spectra established that the vinyl grouping was in the isoprene unit adjacent to the aromatic nucleus, which was consistent with the idea that I contained a phytyl side-chain. In addition, nuclear magnetic resonance spectra provided evidence for the presence of a methoxyl group in IAc. The location of this methoxyl group, on the benzene ring, was defined by mass spectrometry, since the most prominent fragment ion in the mass spectrum of IAc, at m/e 165, could only have arisen if the benzene ring were substituted by the following groups; one methoxyl, one acetoxy, one phytyl, and two methyls. The proposed mass spectral fragmentation scheme for the arbitrarily chosen 1-methoxy-2,3-dimethyl-4-acetoxy-5-phytylbenzene, **1**, well accounts for this ion at m/e 165 (Scheme 1).



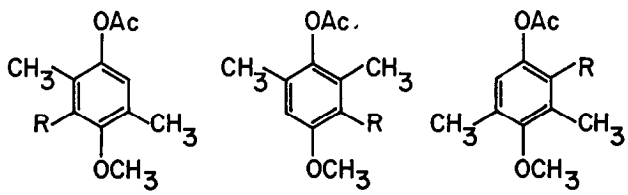
SCHEME 1

This structure, 1-methoxy-2,3-dimethyl-4-acetoxy-5-phytylbenzene, appeared from the first a good possibility for the structure of IAc among the many possible isomers for the pentasubstituted benzene. The *para* orientation of the methoxy and acetoxy groups seemed most likely, since this is the arrangement of the two oxygen atoms in the naturally occurring tocopherols, tocopherol quinones, plastoquinones, and ubiquinones. On comparative grounds, **1** and **2** were most appealing since the arrangement with the two methyl groups adjacent is that found in the plastoquinones and γ-tocopherol known to be present in *Euglena*. In an attempt to solve this orientation problem, we turned to chemical synthesis of the six structural possibilities (**1–6**) (Vance and Bentley, following paper).

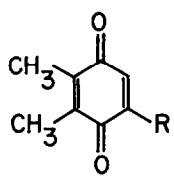
Neither of the chemically synthesized compounds **1** and **2** was identical with the natural compound IAc. The infrared spectra of **1** and **2** were indistinguishable from



1 2 3



4 5 6



7 R = phytyl

that of IAc, and the ultraviolet spectra of IAc (λ_{max} 276 nm, shoulder 285 nm), of **1** (λ_{max} 277, shoulder 269 nm) and of **2** (λ_{max} 277 nm, shoulder 270 nm) were also very similar. The mass spectra of **1** and **2** are summarized in Table 5; on comparison with the mass spectrum of IAc (Table 3) it can be seen that the only differences are small differences in the relative intensities of some ions. The nuclear magnetic resonance

TABLE 5

RELATIVE INTENSITIES OF SOME FRAGMENT IONS FROM THE MASS SPECTRA OF THE SIX SYNTHETIC DIMETHYLPHYTYL-1-METHOXY-4-ACETOXYBENZENES, AND THE ISOMER OF α-TOCOPHEROL ACETATE (IAc)

Compound	% Relative intensity of ion at m/e[a]					
	472	430	207	205	165	43
IAc	5.8	66.7	3.7	5.5	100.0	11.5
1	18.4	100.0	—	44.7	50.0	30.6
2	9.8	46.3	8.9	22.2	100.0	17.4
3	21.0	100.0	14.8	26.8	92.4	33.6
4	5.2	19.4	42.9	1.3	100.0	10.6
5	14.2	100.0	17.4	33.1	37.6	25.5
6	15.6	100.0	33.1	46.1	49.9	29.2

[a] The intensities are relative to the fragment ion of highest intentity.

spectra of **1** and **2** are shown in Table 4; these spectra closely resemble that of IAc except that the τ values for the aromatic methyl groups and the aromatic proton are different. The optical rotations, $[\alpha]_{330}$, of IAc and product **1** were positive and of the same order of magnitude (+7.1 and +6.7 deg cm^2 dg^{-1}, respectively). The three compounds, IAc, **1** and **2**, co-chromatographed on thin-layer chromatography in chloroform and chloroform : benzene 1:1. However, on gas–liquid chromatography (6 ft, 3% OV-1, 250°C) the synthetic product **2** was well separated from IAc (Table VI); the other synthetic product **1**, did not co-chromatograph with IAc either; it chromatographed as a shoulder which eluted slightly before IAc. Therefore, on the basis of gas chromatography and nuclear magnetic resonance spectra we must conclude that IAc has neither structure **1** nor **2**.

Similarly, compounds **3**–**6** were synthesized (Vance and Bentley, following paper) and characterized by ultraviolet, infrared, nuclear magnetic resonance, and mass spectra. The mass spectra, all of which are similar to the mass spectrum of IAc, are summarized in Table 5. Since only a small amount of **4** was synthesized (a by-product of the synthesis of **3**), it could not be isolated free from **3**. However, since **3** and **4** separated on gas chromatography, their mass spectra were obtained. The results of the nuclear magnetic resonance spectra of **3**, **5**, and **6** are recorded in Table 4; although all these spectra were similar, the one which most closely resembled the nuclear magnetic resonance spectrum of IAc, was that of compound **6**. On gas chromatography on 3% OV-1, none of the synthetic compounds **1**–**6** co-chromatographed with IAc. The relative retention times of the six synthetic isomers and IAc are shown in Table 6; each of the synthetic compounds had a shorter retention time than IAc. Therefore, we conclude that IAc is not identical to any of the six synthetic isomers.

TABLE 6

GAS CHROMATOGRAPHIC RETENTION TIMES OF THE SIX SYNTHETIC ISOMERS, RELATIVE TO α-TOCOPHEROL ACETATE ISOMER

Compound	Relative retention time[a]
IAc	1.00
1	0.97
2	0.86
3	0.93
4	0.81
5	0.93
6	0.88

[a] A 6 ft, 3% OV-1 column was used.

In order to rule out completely these six possibilities for the structure of IAc we had to discount one other factor. As we have noted, the nuclear magnetic resonance spectrum of compound **6** was very similar to that of IAc. It was possible that the only difference in structure between IAc and compound **6** was that the arrangement of the groups at the aliphatic double dond was *cis* in the compound **6** and *trans* in IAc (or *vice versa*) which could account for the difference in gas chromatographic behavior. This possibility was eliminated by catalytic hydrogenation of IAc and compound **6**. Although dihydro IAc co-chromatographed with dihydro compound **6** on 3% OV-1 and 3% OV-17 columns, the two hydrogenated products partially separated on a 6 ft, 3% OV-225

column, so that there was a distinct broadening of the peak obtained when the two compounds were coinjected. A comparison of the mass spectra of the two hydrogenated compounds showed significant differences in intensities of some fragment ions; in addition, there were some differences in the mass spectra of IAc and compound **6** (Table 5). These mass spectral data, combined with the slight separation of the two dihydro compounds on gas chromatography, confirmed that IAc did not have structure **6**.

After we had established the major structural features of the new *Euglena* compound (*16*, *17*) including the novel OCH_3 and phytyl groups, the isolation of a "Compound E" from *E. gracilis*, having the molecular formula $C_{29}H_{50}O_2$ (low and high-resolution mass spectrometry) was described by Whistance and Threlfall (*18*). The ultraviolet spectrum of this compound and a positive Emmerie–Engel reaction suggested an isoprenoid *p*-benzoquinol or benzochromanol structure. On oxidation of a small sample (108 μg) with gold chloride, phytylplastoquinone, **7**, was said to be obtained. On the basis of this evidence, and an incorporation of radioactivity from L-[Me-^{14}C]methionine into the metabolite, compound E was characterized as the monomethyl ether of phytylplastohydroquinone. The acetate of this hydroquinone is either structure **1** or **2**.

While there are obvious similarities between compound E and the phenol which we have isolated, the materials differ in some respects.[7] 1. The yields of IAc were routinely 1 mg from 25 g wet weight of cells[8]; however, from 63 g wet weight of cells only 405 μg of compound E were isolated. One possible explanation for this difference is that different growth media were used in the two studies. 2. In our hands, the free phenol appeared unstable on thin-layer chromatography, whereas compound E is 80% recoverable after chromatography in three thin-layer systems in the sequence: adsorptive, reversed phase, adsorptive. 3. We were not able to hydrolyze IAc to the free phenol whereas Whistance and Threlfall have hydrolyzed the acetate of compound E using NaOH in ethanolic pyrogallol. A direct comparison is complicated by the fact that we found it advisable to derivatize the phenol to the acetate, whereas Whistance and Threlfall worked with the free phenol.

Although the structure, monomethylether of phytylplastohydroquinone, is an attractive one on general comparative grounds, our evidence from the synthetic compounds, and their hydrogenation products, is incompatible with it; we have apparently eliminated **1** and **2** for the structure of the acetate of the methoxyphenol on the basis of the gas chromatographic separation. It is also possible that IAc may be one of the six basic structures (**1**–**6**) in which there has been a modification of the usual phytyl side-chain; however, this portion of the molecule must contain the same number of C and H atoms as the phytyl moiety, and must have the double bond in the same position relative to the benzene ring. Other possibilities include the ortho and meta isomers.

[7] One referee of this paper was presumably Dr. Threlfall and some of the statements made in this paragraph are taken from his comments. He believes "it is almost certain that I and compound E are the same compound"; for convenience, the unresolved differences are stated here. We must acknowledge with thanks that the present paper has benefited from Dr. Threlfall's thoughtful suggestions and criticisms made in a detailed and lengthy review.

[8] Other apparently related compounds were present in the extracts. One of these in the 1% ether–petroleum ether fraction from the alumina column was examined in preliminary experiments. After conversion to a trimethylsilyl derivative, this compound had a gas chromatographic retention time of 0.69 relative to the trimethylsilyl derivative of I; for the acetyl derivative, the retention time was 0.70 relative to that of IAc. The mass spectrum of the acetate was like that of IAc but many of the peaks, including the parent molecular ion, were 14 mass units lower, i.e., this phenol is isomeric with β or γ-tocopherol and is a demethyl form of I.

The biological function of these methoxy phenols is not clear. The synthetic compound **1**, was tested as a vitamin E factor in the prevention of muscular dystrophy in rabbits, and found to have no activity. Our survey experiments indicate that compound I does not have a wide distribution in nature, and on the basis of the present evidence it appears to be restricted to *Euglena*.

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